Title
Rational polytherapy in the treatment of cholinergic seizures.

Authors
Niquet, Jerome
Lumley, Lucille
Baldwin, Roger
et al.

Publication Date
2020

DOI
10.1016/j.nbd.2019.104537

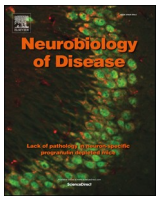
Peer reviewed

Neurobiology of Disease xxx (xxxx) xxxx

Contents lists available at ScienceDirect

Neurobiology of Disease

journal homepage: www.elsevier.com/locate/ynbdi



Review

Rational polytherapy in the treatment of cholinergic seizures

Jerome Niquet[a,b], Lucille Lumley[c], Roger Baldwin[b], Franco Rossetti[d], Lucie Suchomelova[b], David Naylor[a], Ireri Betsabe Franco Estrada[a,b], Mark Schultz[c], Marcio de Araujo Furtado[c,d], Claude G. Wasterlain[a,b,e,*]

[a] Department of Neurology, David Geffen School of Medicine at UCLA, Los Angeles, CA, USA
[b] Epilepsy Research Laboratory (151), Veterans Affairs Greater Los Angeles Healthcare System, Los Angeles, CA, USA
[c] Neuroscience Department, US Army Medical Research Institute of Chemical Defense (USAMRICD), 8350 Ricketts Point Rd., Aberdeen Proving Ground, MD 21010, USA
[d] Military Psychiatry and Neuroscience Department, Walter Reed Army institute of Research, Silver Spring, MD, USA
[e] Brain Research Institute, David Geffen School of Medicine at UCLA, Los Angeles, CA., USA

A B S T R A C T

The initiation and maintenance phases of cholinergic status epilepticus (SE) are associated with maladaptive trafficking of synaptic $GABA_A$ and glutamate receptors. The resulting pharmacoresistance reflects a decrease in synaptic $GABA_A$ receptors and increase in NMDA and AMPA receptors, which tilt the balance between inhibition and excitation in favor of the latter. If these changes are important to the pathophysiology of SE, both should be treated, and blocking their consequences should have therapeutic potential.

We used a model of benzodiazepine-refractory SE (RSE) (Tetz et al., 2006) and a model of soman-induced SE to test this hypothesis. Treatment of RSE with combinations of the $GABA_AR$ agonists midazolam or diazepam and the NMDAR antagonists MK-801 or ketamine terminated RSE unresponsive to high-dose monotherapy with benzodiazepines, ketamine or other antiepileptic drugs (AEDs). It also reduced RSE-associated neuronal injury, spatial memory deficits and the occurrence of spontaneous recurrent seizures (SRS), tested several weeks after SE. Treatment of sc soman-induced SE similarly showed much greater reduction of EEG power by a combination of midazolam with ketamine, compared to midazolam monotherapy.

When treating late (40 min after seizure onset), there may not be enough synaptic $GABA_AR$ left to be able to restore inhibition with maximal $GABA_AR$ stimulation, and further benefit is derived from the addition of an AED which increases inhibition or reduces excitation by a non-GABAergic mechanism. The midazolam-ketamine-valproate combination is effective in terminating RSE. 3-D isobolograms demonstrate positive cooperativity between midazolam, ketamine and valproate, without any interaction between the toxicity of these drugs, so that the therapeutic index is increased by combination therapy between $GABA_AR$ agonist, NMDAR antagonist and selective AEDs.

We compared this drug combination based on the receptor trafficking hypothesis to treatments based on clinical practice. The midazolam-ketamine-valproate combination is far more effective in stopping RSE than the midazolam-fosphenytoin-valproate combination inspired from clinical guidelines. Furthermore, sequential administration of midazolam, ketamine and valproate is far less effective than simultaneous treatment with the same drugs at the same dose. These data suggest that we should re-evaluate our traditional treatment of RSE, and that treatment should be based on pathophysiology. The search for a better drug has to deal with the fact that most monotherapy leaves half the problem untreated. The search for a better benzodiazepine should acknowledge the main cause of pharmacoresistance, which is loss of synaptic $GABA_AR$. Future clinical trials should consider treating both the failure of inhibition and the runaway excitation which characterize RSE, and should include an early polytherapy arm.

1. Introduction

Because of the ease with which they are synthesized, concealed and of their potential for mass casualties, nerve agents are among the most serious terrorist threats to day. Military stockpiles reach gigantic levels in many countries. The potential terrorist threat is even greater, because agents such as sarin can easily be synthesized, transported, concealed and of the potential availability of some military stockpiles to terrorists. Seizures are the most treatment-refractory complication of nerve agent intoxication, and were a prominent feature in the Tokyo subway attacks (Nakajima et al., 1998) and in the recent use of nerve agents in Syria. They turn into uncontrolled status epilepticus (SE), refractory to treatment with antiepileptic drugs and cause severe brain damage (Shih et al., 1999, McDonough Jr. and Shih, 1993) and chronic epilepsy (de Araujo Furtado et al., 2010). Finding a treatment for those seizures is clearly an important national goal. In addition, the insights and practical applications derived from this study are likely to find applications in the treatment of organophosphate insecticide poisoning, which occurs through agricultural or voluntary (attempted suicide) exposure throughout the world, and in the treatment of status epilepticus (SE), a condition affecting 102–152,000 cases/year in the USA, with an estimated 22–42,000 deaths yearly (DeLorenzo et al., 1996).

* Corresponding author at: Neurology Service (127), VA Greater Los Angeles Healthcare System, 11301 Wilshire Boulevard, West Los Angeles, CA 90073, USA.
E-mail address: wasterla@ucla.edu (C.G. Wasterlain).

https://doi.org/10.1016/j.nbd.2019.104537
Received 18 March 2019; Received in revised form 25 June 2019; Accepted 18 July 2019

While some aspects of nerve agent-induced SE are unique, its classical features have much in common with other forms of SE (Chen and Wasterlain, 2006). It becomes self-sustaining, independent of its original cholinergic trigger, pharmacoresistant to benzodiazepines (Shih et al., 1999), and remains relatively responsive to NMDA blockers (Dorandeu et al., 2019), but these features are shared by SE induced by perforant path stimulation (Mazarati et al., 1998a, 1998b, Mazarati and Wasterlain, 1999), amygdala stimulation (Nissinen et al., 2000), or lithium and pilocarpine (Morrisett et al., 1987; Suchomelova et al., 2006). It is probably shared by human SE, where the time of treatment is a better determinant of therapeutic response than the drug used (Treiman et al., 1998). The therapeutic lessons of this study are likely to apply to these other forms of SE as well.

While we have made considerable progress in treating nerve agent intoxication, their tendency to produce severe seizures and SE remains a therapeutic challenge. These seizures quickly become self-sustaining, independent of the cholinergic network, and refractory to anticonvulsants (Shih et al., 1999; Kapur and Macdonald, 1997; Mazarati et al., 1998b). They display reduced GABAergic inhibition (Kapur et al., 1989, Rice and DeLorenzo, 1999, Naylor and Wasterlain, 2005a, 2005b) and enhanced glutamatergic excitation (Kapur and Lothman, 1990; Wasterlain et al., 2000; Naylor et al., 2013). Our studies and others, using a cholinergic SE model induced by lithium-pilocarpine, suggest that seizures cause internalization (and inactivation) of synaptic $GABA_A$ receptors ($GABA_AR$) (Naylor et al., 2005; Goodkin et al., 2008), and promote movement of NMDA receptor (NMDAR) subunits to synapses where they form additional functional receptors (Naylor et al., 2013) and NMDA-dependent movement of AMPA receptors (AMPAR) to synapses (Rajasekaran et al., 2012). It should be noted that the changes in AMPAR described in SE are NMDAR-activation-dependent, so that blocking NMDAR may block the changes induced by both NMDAR and AMPAR.

The loss of synaptic $GABA_A$ receptors and the increase in synaptic NMDAR and AMPAR are proconvulsant and maintain seizure activity. This might explain why muscarinic antagonists, which are very effective in blocking seizure development when given early during nerve agent intoxication, cannot stop the seizures once they have become established. Blocking muscarinic receptors does not correct the reduced number of synaptic GABAAR or the increased number of synaptic NMDAR and AMPAR, it amounts to closing the barn door after the horses have escaped. This suggests that targeting a single mechanism (e.g. $GABA_AR$) is unlikely to be successful, and that polytherapy is needed to 1/stimulate the remaining $GABA_AR$; 2/reduce the activity of NMDAR; 3/enhance inhibition by a non-GABA, non-NMDA mechanism, since strategy number 1/, while useful, cannot fully restore inhibition if the reduction of the number of synaptic $GABA_AR$ is severe. The choice of the third drug is beyond the scope of this review. Valproate, a drug with an unknown mechanism of action and no affinity for $GABA_AR$, is one of several antiepileptic drugs (AEDs) which we found to be effective.

Since we have not been successful at blocking seizure-induced receptor trafficking in vivo, we examined whether blocking its consequences might achieve the same result. Indeed, treatment of cholinergic seizures with combinations of drugs which enhance $GABA_AR$ mediated inhibition, and block NMDAR mediated excitation proved very effective in the treatment of cholinergic SE. Using this approach, we can stop severe cholinergic seizures using subanesthetic doses of drugs while limiting toxicity. Nerve agents and organophosphate insecticides inhibits acetylcholinesterase (Ache), and have both muscarinic and nicotinic effects. Seizures are prevented by early administration of atropine, scopolamine and muscarinic blockers, and untouched by nicotinic antagonists (McDonough Jr. and Shih, 1993), suggesting that the muscarinic component is responsible for the majority of ictogenic effects. This led to the development of models based on the administration of lithium and pilocarpine (Tetz et al., 2006) to mimic seizures induced by soman. We used that model in most of our studies. The most effective drug combinations in this model were then tested in a model of SE induced by subcutaneous injection of the nerve agent soman (also named GD). The similarity of therapeutic results in these two seizure types suggests that the high-dose lithium-pilocarpine model of SE is highly predictive of nerve agent-induced SE.

2. Methods

The detailed methods have been published (Niquet et al., 2016a,b; Niquet et al., 2017a,b; Schultz et al., 2014) and will be described briefly.

2.1. Animals

Male Sprague-Dawley rat (200–300 g, mean 249 g; Charles River, MA) were housed in a temperature- and humidity- controlled room with 12 h light-dark cycles and had free access to food and water. All experiments were conducted with the approval and in accordance with the regulations of the Institutional Animal care and Use Committee of West Los Angeles VA Medical Center. For the soman studies, male Sprague-Dawley rats (350–400 g; Charles River) were housed on 12 h reverse light-dark cycle. The experimental protocol was approved by the Animal Care and Use Committee at both the United States Army Medical Research Institute of Chemical Defense and all procedures were conducted in accordance with the principles stated in the Guide for the Care and Use of Laboratory Animals, and the Animal Welfare Act of 1966 (P.L. 89-544), as amended.

2.2. Induction of SE, monotherapy and polytherapy

Rats were administered lithium chloride (5 mEq/kg) subcutaneously and, 16 h later, SE was induced with i.p. pilocarpine hydrochloride (320 mg/kg). Only rats displaying behavioral/EEG seizures were used. All rats received scopolamine methyl bromide (1 mg/kg; i.p.), a muscarinic antagonist that does not cross the blood-brain barrier, at the same time as pilocarpine, to decrease peripheral cholinergic side-effects such as bronchial secretions. Seizures occurred 7.6 ± 2.7 min after pilocarpine injection, so that time from pilocarpine injection to mono or dual therapy was approximately 48 min. All animals subsequently received scopolamine (10 mg/kg i.p.) to remove the original seizure trigger without stopping SE, and sham injection (control SE group), one drug (monotherapy) or a combination of drugs (polytherapy) i.p. 40 min after EEG seizure onset. Drugs included midazolam, ketamine and sodium valproate. The valproate group was used as an additional "control" group because mortality rate in the SE control group was high, and valproate monotherapy did not measurably alter seizure activity. Note that monotherapy groups have double or triple dose of drugs compared to polytherapy groups to compensate for the total amount of drugs. For long-term behavioral studies, a sham group, which did not receive drug treatment and was not exposed to SE, was added.

Rats not capable of coordinated walking and movement 16 h after SE were injected SC (10 ml/kg) with 5% glucose twice per day until capable of coordinated movement or until euthanasia at three days. Water moistened food pellets and/or gelatin cubes were placed in the cage in Petri dishes. Euthanasia criteria consisted of failure to achieve coordinated movement three days after SE. Animals were also euthanized if showing a weight loss of 5% sustained over two days after the coordination criterion had been achieved.

2.3. Soman exposure

Rats were subcutaneously (s.c.) administered 1.2 LD50 GD (pinacolyl methylphosphonofluoridate; 132 μg/kg; 0.5 ml/kg) obtained from Edgewood Chemical Biological Center (Aberdeen Proving Ground, MD, USA) and 1 min later administered an intramuscular (i.m.) injection

with an admix of atropine sulfate (2 mg/kg) and the oxime HI-6 dimethanesulfonate salt (93.6 mg/kg). Forty minutes after seizure onset, drugs (midazolam, ketamine or sham injection) were administered i.p. as either a monotherapy or a polytherapy.

2.4. Implantation of electrodes

Under isoflurane anesthesia, the animals were implanted with stainless steel skull screws to serve as recording electrodes. Two electrodes were used for bipolar recording and were located 3 mm anterior to lambda and 4 mm left and right of the medial suture. The third electrode served as reference and was located 1 mm anterior to bregma and 1 mm to the right of the mid-line defined by the medial suture. The screw electrodes were connected to a tri-polar connector (Plastics One, VA) and dental cement was used to cover the electrodes so that only the connector was exposed. Animals were used one to two weeks after electrode implantation. The BioPac Systems MP150 was used to record digital EEG using a BioPac UM100A preamplifier. Sampling rate was 200 Hz.

For soman studies, rats were implanted with F40-EET telemetry transmitters (Data Sciences International, Inc.) as described in Schultz et al., 2014 and received continuous EEG recording.

2.5. Acute video-EEG monitoring

Recording was started before agent (pilocarpine or soman) exposure and was continuous for 24 h. For pilocarpine EEG analysis, the EEGs were processed offline to detect seizures and spikes using Stellate Systems Harmony software (Natus) with default parameters: amplitude threshold 2.7, minimum frequency 3 Hz, maximum coefficient of variation 40% for seizure detection and a spike amplitude threshold of 6 for spike detection. For soman EEG analysis, epileptiform activity was identified using Dataquest ART 4.1 (analysis software), Neuroscore 1.1.1 (Data Systems International, Arden Hills, MN), and a customized MATLAB (release 2008a, Mathworks, Natick, MA) algorithm according to de Araujo Furtado et al. (2009) and confirmed by visual screening., The outcome measures were the ratio of EEG power integral over a defined period of time divided by the average baseline EEG power before exposure; the number of seizures per 24 h, the cumulative seizure time per 24 h (time spent seizing, subtracting post- and inter-ictal time); the number of spikes per 24 h; and the time needed for EEG amplitude to fall for the first time below 2 times the pre-pilocarpine EEG amplitude and be free of semi-periodic spikes or sharp waves for at least 1 min, which in this experimental paradigm is close to the time of termination of SE.

2.6. Tissue preparation for detection of acute neuronal injury

The animals were anesthetized with an overdose of pentobarbital (100 mg/kg i.p.) 48 h after induction of SE, and after transcardiac perfusion with 4% phosphate-buffered formaldehyde brains were kept in situ at 4 °C overnight, postfixed in the same perfusate for 2–3 h. and kept in PB 0.1 M containing 30% sucrose for 48–72 h. Floating sections (30 μM thickness) were obtained using a sliding microtome. Coronal sections were stained with fluoro-Jade B. When needed, the number of injured cells was counted by unbiased stereology using the optical dissector method.

2.7. Recording of spontaneous recurrent seizures (SRS)

For chronic recordings, rats were implanted 4 weeks after pilocarpine-induced SE and monitored 2 weeks later for 2 weeks (24/7) with a digital video-EEG system. Electrographic seizures were analyzed off-line and seizures were confirmed by manual review of the tracing morphology and of the digital videos. Outcome measure was the average number of SRS per week.

2.8. Morris water maze paradigm

In pilocarpine exposed rats, spatial learning and memory were evaluated one week after SRS recording with a modified Morris water maze paradigm, by requiring the rats to swim in a pool 170 cm in diameter, with the water kept at 260 °C, to find a 12-cm diameter circular platform submerged 2 cm beneath the surface of the water, which was opacified by the addition of black non-toxic tempera paint. The platform was in a constant position during training, as there were a number of visual cues in the testing room. Experiments were monitored with a Sony CCD-IRIS high-resolution camera mounted above the pool and using indirect lighting from a 25 W bulb. A video-tracking system (Ethovision; Noldus, Inc. Wageningen, The Netherlands) was used for data acquisition. The rats were brought to the experimental room at least 30 min prior to an experiment. Each rat was trained to find the hidden platform kept in the same location for one session of eight trials per day, for 5 consecutive days. The start sequence was randomly selected and was different for each day. For training, the rat was released in the water from one of the four starting positions, facing the wall of the pool. It was given 60s to locate and climb onto the platform, where it stayed for 30s. If a rat did not find the platform within 60s, it was gently guided to it by experimenter. After the end of the last session, the rat was dried with absorbent paper and kept in a warm cage. Ten days later, retention test for long-term memory retention was performed.

Soman-exposed rats were evaluated one month after exposure for spatial memory acquisition in the Morris water maze using a video-tracking program (HVS Watermaze 2100, HVS Image, Cambridge, UK), using a similar paradigm to that described above. For detailed methods, see Schultz et al., 2014.

2.9. Toxicity studies

The following scale was used: 0 normal gait; 1 ataxic; 2 unable to walk but able to crawl, righting reflex preserved; 3 righting reflex partially impaired; 4 complete loss of righting reflex; 5 no response to tail pinch; 6 loss of corneal reflex. The toxicity score was the sum of measures taken every 15 min for the first hour after injection.

2.10. Construction of 3-dimensional isobolograms for toxicity and efficacy

We defined the ED50 as the dose which reduced a particular measure of seizure severity by 50%, and the TD50 as the dose which induced half-maximal toxicity. Dose-response curves were fit by the method of Chou (2006).10 The variances of the TD50s were calculated by the method of Tallarida (p 29, equation 2.9).11 The theoretical dosage sum was calculated from Tallarida (p 59, equation 4.1, and its variance from equation 4.2, p 60). For each isobologram, a plane was fit through the ED50s or TD50s using SciDavis (http://scidavis, sourceforge.net). The observed toxicity or efficacy of the triple drug combination was then placed in the same 3 dimensional plot with the plane connecting TD50s or ED50s for individual drugs. Drug synergism (positive cooperativity) is indicated if the observed toxicity or efficacy of the combination lies below the additivity plane in the isobolograms. When the effects are simply additive, the values fall in the additivity plane. Negative cooperativity would result in values above that plane. Significance was determined by a modified *t*-test (Tallarida, Section 2.3, p 60–62).11

2.11. Statistical analyses

EEG and cell injury data often showed a non-Gaussian distribution and were analyzed with nonparametric statistical methods: Kruskal-Wallis test followed by Dunn's multiple comparison test (GraphPad version 6). MWM data was analyzed by 2-way ANOVA (GraphPad version 6). Kaplan-Meier analysis was used for comparison of soman induced SRS. Statistical significance was defined as $p < 0.05$. In all

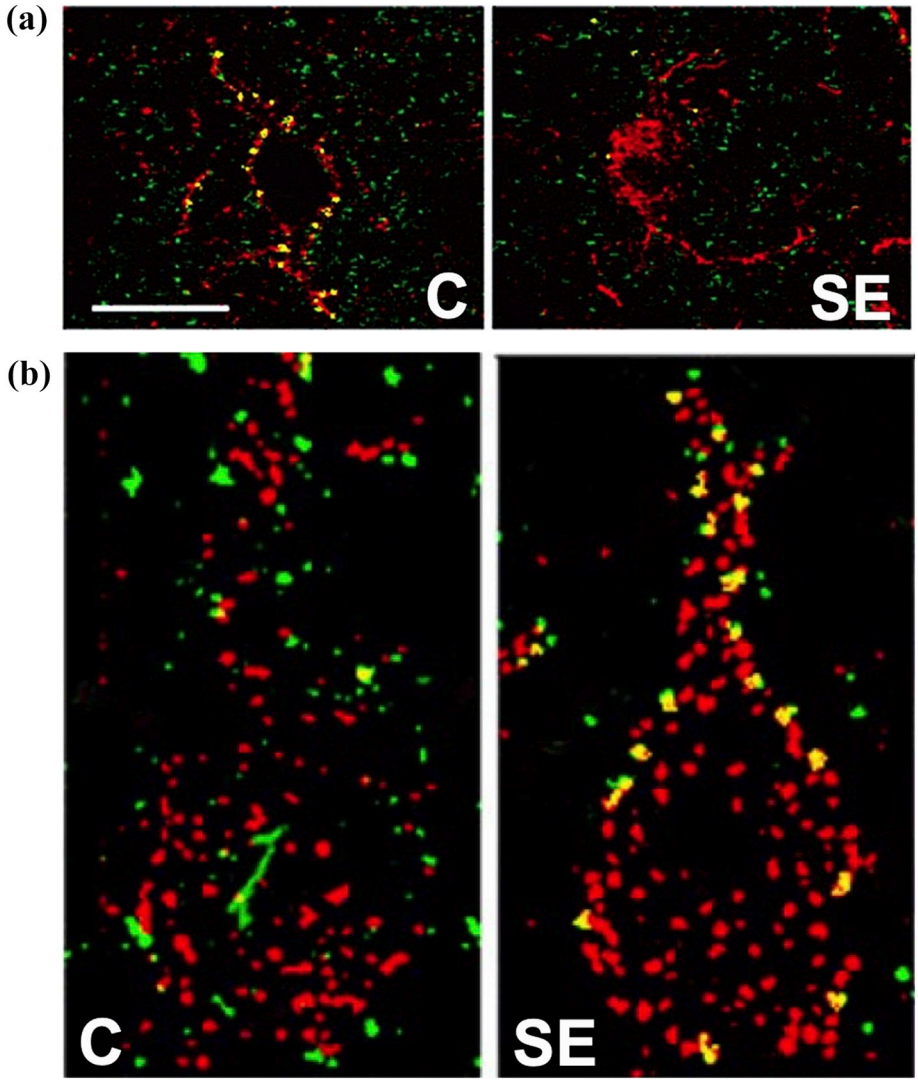


Fig. 1. A) In a control dentate gyrus, the γ2 subunits of GABAA R (red pseudocolor, left) and the synaptic marker synaptophysin (green pseudocolor) colocalize (yellow colour) on synapses on the somatic surface and proximal dendrites of dentate granule cells, but after 1 h of SE induced by lithium 3 mEq/kg and pilocarpine 100 mg/kg, many are internalized in the cytoplasm, and there are fewer co-localizations. B) The opposite happens to NR1 subunits of the NMDAR (red pseudocolor, right) which are abundant in the cytoplasm of controls, but during SE move to the neuronal surface where they colocalize (yellow dots) with synaptophysin (green). C) On the left, we recorded $GABA_A$ miniature inhibitory synaptic currents (mIPSCs), which under the conditions of the experiment reflect the response of post-synaptic $GABA_AR$ to a quantum of GABA released from a single synaptic vesicle. mIPSCs on the soma of dentate granule cells are of lower amplitude in the slice prepared from a rat in lithium-pilocarpine SE for 1 h (red line) than in the control slice (black line). Based on a seven-state model of the $GABA_AR$ (Naylor et al., 2005), this indicates a reduction in the number of $GABA_AR$ per synapse (C: 36 ± 11 $GABA_AR$/synapse, SE: 18 ± 4 $GABA_AR$/synapse, $p < 0.001$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
In the center, we recorded miniature excitatory NMDA post-synaptic currents (NMDA mEPSCs) from dentate granule cells under similar conditions, reflecting the response of post-synaptic NMDA receptors to a quantum of glutamate released from a single vesicle. Here, the amplitude of the post-synaptic response to a quantum of glutamate is greater in the slice from a rat in SE (red line) than in a control (black line). Based on the model of the NMDAR used by Naylor et al. (2013), this indicates an increase in number of NMDA receptors per synapse in SE (C: 8 ± 1 NMDAR/synapse, SE: 11 ± 2 NMDAR/synapse, $p < 0.001$). On the right, we recorded miniature excitatory AMPA/Kainate miniature post-synaptic currents (Non-NMDA mEPSCs) from dentate granule cells under similar conditions, reflecting the response of post-synaptic AMPA/Kainate receptors to a quantum of glutamate released from a single vesicle. The amplitude of the postsynaptic response is slightly but significantly greater in the slice from a rat in SE (red line) than in a control (black line), indicating an increased response of post-synaptic AMPAR and/or Kainate receptors to a quantum of glutamate in SE, due to increased number or increased activity of these receptors. Here, we did not have immunocytochemistry to differentiate between these two options. AMPA/Kainate-mEPSCs amplitude increased from −10.8 ± 1.2 pA in controls to −12.8 ± 1.6 pA after SE ($p < 0.001$). There was no change in the single channel conductance (13 ± 2 pS and 12 ± 2 pS for C and SE, respectively; n.s.), but an increase in the number of AMPA/Kainate receptors per synapse from 32 ± 6 to 39 ± 4 ($p < 0.001$). AMPA/Kainate-mEPSCs also have an increased tau decay from 5.01 ± 0.63 to 5.66 ± 0.76 ($p < 0.05$) with SE, but no significant change in event frequency.
Modified from Naylor et al., 2005, 2013.

graphs, data are presented as median values with the interquartile range, which is the difference between the 75th and 25th percentile. When data were normally distributed, we used mean ± SEM.

3. Results and discussion

3.1. Biological basis for treatment of cholinergic SE with drug combinations

The transition from single seizures to SE (the initiation phase of SE) and the maintenance of self-sustaining seizures (the maintenance phase of SE) have been extensively studied in the lithium-pilocarpine model of SE, in nerve agent-induced SE, and in non-cholinergic models, which we will not review. In cholinergic animal models, SE is associated with three key changes: 1/ internalization of synaptic $GABA_AR$ (Fig. 1A) coupled with a decrease in $GABA_A$ mIPSCs (Fig. 1C) indicating a reduction of the number of post-synaptic $GABA_AR$ per synapse (Naylor et al., 2005). This may represent a seizure-induced acceleration of the normal process of receptor trafficking, in which synaptic $GABA_AR$ are internalized into endosomes and transiently inactivated, and can later be recycled to the Golgi apparatus and to the synaptic membrane (Terunuma et al., 2008; Nakamura et al., 2015). Interestingly, extrasynaptic $GABA_AR$, which have high affinity for GABA and do not desensitize easily (Ferando and Mody, 2012), are not internalized during SE, making them potential therapeutic targets (Naylor et al., 2005). 2/ Increased synaptic localization of NMDAR (Fig. 1B), coupled with an increase in NMDA mEPSCs (Fig. 1C) reflecting an increased number of NMDAR per synapse, presumably representing assembly and movement to the membrane of spare NMDAR subunits. 3/ Increased AMPA/Kainate synaptic currents after SE in dentate granule cells (Naylor and Wasterlain, 2005a, 2005b), and NMDAR-dependent increased surface localization of GluA1, but decreases in GluA2 AMPAR subunits in CA1, but not in DG (Rajasekaran et al., 2012, Joshi and Kapur, 2018), presumably reflecting assembly and movement to the membrane of spare GluA1 subunits. All three of those changes are maladaptive. The reduction of post-synaptic $GABA_AR$ can lead to failure of inhibition, and the increase of post-synaptic NMDAR and AMPAR can lead to runaway

excitation.

The receptor trafficking hypothesis states that seizure-induced receptor trafficking (loss of synaptic $GABA_AR$ and increase in synaptic ionotropic glutamate receptors) is a key factor in the development of SE-associated pharmacoresistance. This does not rule out a role for the many other physiological changes which are known to occur during SE, including changes in transmitter release. However, it suggests that blocking receptor trafficking or its consequences has the potential to overcome that pharmacoresistance and to make seizures responsive to drug treatment even when that treatment is delayed.

At present, we cannot prevent SE-associated receptor trafficking, but we can block its consequences. We can stimulate remaining synaptic GABAAR with benzodiazepines or other GABAergic drugs. If treatment is late and too few GABAAR are left in synapses to fully restore inhibition by that mechanism, we can add a drug which increases inhibition by a non-GABAergic mechanism (or reduces excitation). And we can use NMDAR and AMPAR blockers to reduce excitation. Combining GABAAR agonist and NMDAR antagonist drugs in the initial treatment of SE is not recommended by current therapeutic guidelines (Glauser et al., 2016), but is effective in the high-dose lithium/pilocarpine model (Tetz et al., 2006) and in a soman model of cholinergic SE, as shown below.

3.2. Synergistic effects of the GABAAR agonist – NMDAR antagonist combination in treating benzodiazepine-refractory cholinergic SE (RSE)

Trafficking of GABAA and ionotropic glutamate receptors is one of many changes which occur at the transition from single seizures to SE. If they are indeed the key to the initiation and maintenance of SE, treatment which blocks these changes should stop SE. Indeed, previous studies have suggested a synergistic seizure-stopping interaction between diazepam and the NMDAR channel blocker ketamine (Martin and Kapur, 2008). We treated cholinergic SE with a combination of a benzodiazepine (diazepam or midazolam) and an NMDAR antagonist (dizocilpine or ketamine).

3.2.1. High-dose lithium-pilocarpine model

We used MK-801 (dizocilpine), a potent and specific, non-subunit selective NMDA antagonist, to test the principle that a combination of a benzodiazepine with an NMDA antagonist would be superior to monotherapy with ei mortality (1/11 death/24 h vs 12/21 controls) but not duration of SE, cumulative seizure time, time to the first seizure-free minute or Hjorth function (Hjorth, 1991). Moderate to high doses of the $GABA_A$-enhancing benzodiazepine diazepam (5–20 mg/kg) reduced mortality (0/18) but did not affect duration of SE, seizure number, time to the first seizure-free minute, cumulative seizure time or Hjorth function. However, the combination of diazepam with dizocilpine was very effective in stopping SE, eliminating mortality (0/8) and reducing the duration of SE over 100-fold compared to the untreated and diazepam groups (Fig. 2A). Compared to the same groups, it also reduced the latency to the first seizure-free minute of EEG 74-165-fold, and Hjorth function at 1 h (a measure of the severity of the initial seizures which is influenced by EEG power) 10–14-fold (both $p < 0.05$). These results support the hypothesis that cholinergic SE utilizes circuits involving $GABA_A$ and NMDA receptors, and that cholinergic SE-associated changes in number of available synaptic NMDAR play an important role in the maintenance of seizures. See (Fig. 2B).

Since dizocilpine is not approved for human use, we examined whether similar results could be obtained with the low-affinity NMDA channel blocker ketamine, which has been in clinical use for many years, is available in injectable form and is well-absorbed intramuscularly (important for treating mass casualties), has an excellent safety record for anesthesia, and has been used successfully for the treatment of refractory SE (Sheth and Gidal, 1998; Mewasingh et al., 2003; Bleck, 2006; Holtkamp et al., 2005). The combination of low-dose ketamine (10 mg/kg) with moderate dose diazepam (5 mg/kg) reduced the duration of SE six-fold, and the number of seizures 9-fold compared to the control, diazepam 20 mg/kg and diazepam 5 mg/kg groups ($p < 0.001$), and also reduced the delay to the first seizure-free minute of EEG, and Hjorth function, a measure highly correlated to EEG power, over the first hour post-treatment. Hjorth function was also reduced over the first 6 hours post-treatmernt, indicating that seizures did not recur. It also decreased EEG power integral over the first hour following treatment, while EEG power immediately before treatment was the same in all groups (Niquet et al., 2016a,b). Some of ketamine's actions are independent of the NMDAR (Zanos et al., 2016, 2018), but here the effect on SE was probably NMDAR-mediated, since dizocilpine, a fairly selective blocker of the NMDAR ion channel, had similar actions. It should be noted that not all drug combinations are efficacious. In additional experiments, double-dose midazolam monotherapy and double-dose ketamine monotherapy, and dual therapy combining midazolam with valproate or ketamine with valproate decreased EEG power slightly compared to untreated SE, but none of them stopped SE (Niquet et al., 2016a,b). By contrast, the combination midazolam-ketamine decreased EEG power integral below the pre-pilocarpine baseline, showing that seizures stopped (Niquet et al., 2016a,b). These results confirm previous observations and suggest that a $GABA_AR$ agonist-NMDAR antagonist combination has synergistic actions in the treatment of cholinergic SE.

We attempted to determine NMDAR subunit specificity by using NMDAR blockers which are selective for its GluN2A or GluN2B subunits. However, combinations of midazolam with the relatively selective GluN2A blocker TCN201 were poorly effective in stopping RSE. Combinations of midazolam with the GluN2B blockers ifenprodil, felbamate or Ro25–6981 were also less effective than combinations which included dizocilpine or ketamine in blocking RSE 40 min after seizure onset. More detailed studies are needed to resolve the NMDAR subtype involved in the therapeutic actions described in Fig. 2A.

3.2.2. Soman-induced SE

Rats treated with midazolam (3 mg/kg) 40 min after SE had greater 24 h survival (90%) compared to rats treated with saline or with 1 mg/kg midazolam (50% and 60% respectively). The values of EEG power integral of treatment groups were compared in specific time periods (during pre-treatment SE, and during the first hour or the first 6 h after treatment). EEG power integral (a measure of EEG seizure severity) increased in all GD-exposed rats during SE compared to rats that were not exposed to GD, and there were no differences between groups. During the first hour after treatment, rats that received midazolam had a significant reduction of EEG power compared to those that received only vehicle, but by 6 h after exposure there was no difference between these groups (data not shown). Rats treated with ketamine 30 mg/kg were similar to midazolam-treated animals. Rats treated with 30 mg/kg ketamine + 3 mg/kg midazolam after 40 min of GD-induced SE had reduced EEG power integral (reduced seizure severity) during the 1 h after treatment. In addition, during the 1 h and the first 6 h time periods after treatment, rats treated with ketamine - midazolam combination had a greater decrease in percent EEG power integral (70%) compared to midazolam monotherapy (14%) ($p < 0.01$ at 1 h, $p < 0.05$ at 6 h). The first hour is the critical time to reduce neuronal injury (which appears within 20 min. of SE onset) and pharmacoresistance, which increases with seizure burden (Niquet et al., 2016a,b). Higher doses of ketamine (not shown) did not provide additional therapeutic benefits but increased mortality.

3.2.3. Effect of GABAAR agonist-NMDAR antagonist combinations on long-term consequences of cholinergic SE

3.2.3.1. Neuronal injury. No neuronal injury was detected in any ketamine-treated animals in CA3 (Fig. 3a) or CA1, suggesting an NMDAR-dependent mechanism of injury in hippocampal pyramids. Rats treated with the midazolam – ketamine – valproate combination, which stopped RSE rapidly, showed remarkable reduction of neuronal

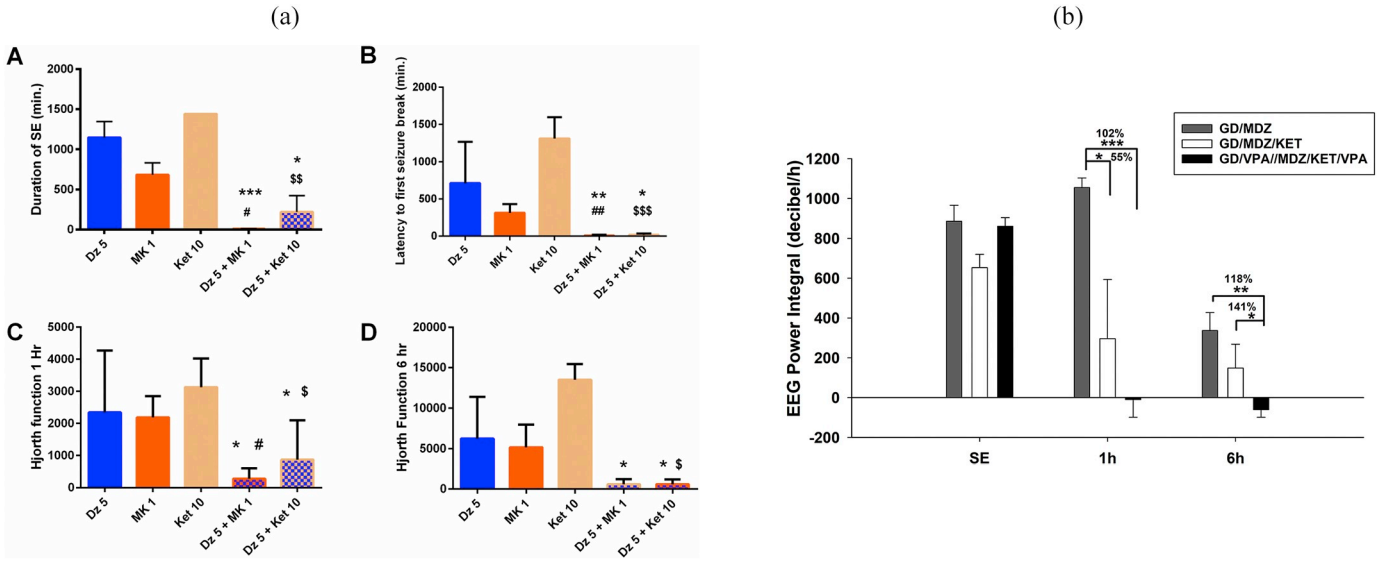


Fig. 2. A) Response of high-dose lithium-pilocarpine RSE to monotherapy with the GABAAR agonist diazepam or the NMDAR antagonists dizocilpine or ketamine, and to combination therapy with GABAAR agonists coupled with NMDAR antagonists. Cholinergic RSE is terminated by GABAAR agonist – NMDAR antagonist combinations, and fails to stop with monotherapy, even at high dose (diazepam 20 mg/kg, not shown). Dz5: diazepam 5 mg/kg; MK1: dizocilpine 1 mg/kg; Ket 10: ketamine 10 mg/kg; Dz5 + MK1: diazepam 5 mg/kg combined with dizocilpine 1 mg/kg; Dz5 + Ket 10: diazepam 5 mg/kg combined with ketamine 10 mg/kg. *, **, ***: $p < 0.05$. 0.01 or 0.001 compared to Diazepam 5 mg/kg. #, ##: $p < 0.05$ or 0.01 compared to dizocilpine 1 mg/kg. $,$$,$$$: p,0.05, 0.01 or 0.001 compared to ketamine 10 mg/kg. Values are median ± interquartile range. Kruskall-Wallis with Dunn's multiple comparisons. B) shows the results of treating sc soman-induced SE with benzodiazepine monotherapy, with ketamine monotherapy, or with a GABAAR agonist-NMDAR antagonist combination, 40 min after seizure onset. EEG power before treatment (SE) was similar in all experimental groups. Treatment with midazolam 3 mg/kg (GD/MDZ/SAL) or with ketamine 30 mg/kg reduced EEG power slightly compared to untreated animals. The midazolam 3 mg/kg / ketamine 30 mg/kg combination (MDZ/KET) reduced the EEG power integral much better than midazolam or ketamine monotherapy. ***$p < 0.001$; **$p < 0.01$; $p < 0.05$. Data shown are mean ± S.E.M. These results suggest that the midazolam-ketamine combination is efficacious in reducing EEG seizure severity against soman-induced SE.

injury, but rats treated with triple-dose ketamine alone, which was poorly effective in stopping EEG seizures and did not terminate RSE, showed equally good protection. Thus this appeared to be related to the neuroprotective properties of ketamine, rather than to the ability of NMDAR antagonist-containing combinations to stop RSE. This has therapeutic implications: it suggests that the early use of ketamine for neuroprotection, regardless of or in addition to its ability to stop seizures, might be a useful strategy in RSE and SRSE. It would be interesting to retrospectively look at MRI evidence of brain damage and brain atrophy in the clinical use of ketamine for RSE, although it is often used so late in the course of RSE that most vulnerable neurons might have been lost before treatment was initiated.

This neuroprotection is all the more remarkable because treatment was administered after 40 min of EEG seizures, and signs of neuronal injury have been documented to occur within 20 min of seizure onset in a model of lithium – pilocarpine SE milder than the model we used (Fujikawa, 1996). It is likely that ketamine treatment reversed neuronal injury in hippocampal principal neurons, in spite of its delayed delivery. The fact that ketamine neuroprotection was less complete in dentate hilus than in CA1 or CA3 was expected, and suggests that neuronal injury in that area is less dependent on NMDAR activation than in CA1. It confirms that the mechanisms of neuronal injury from the same seizures can vary with the brain region (Lopez-Meraz et al., 2010).

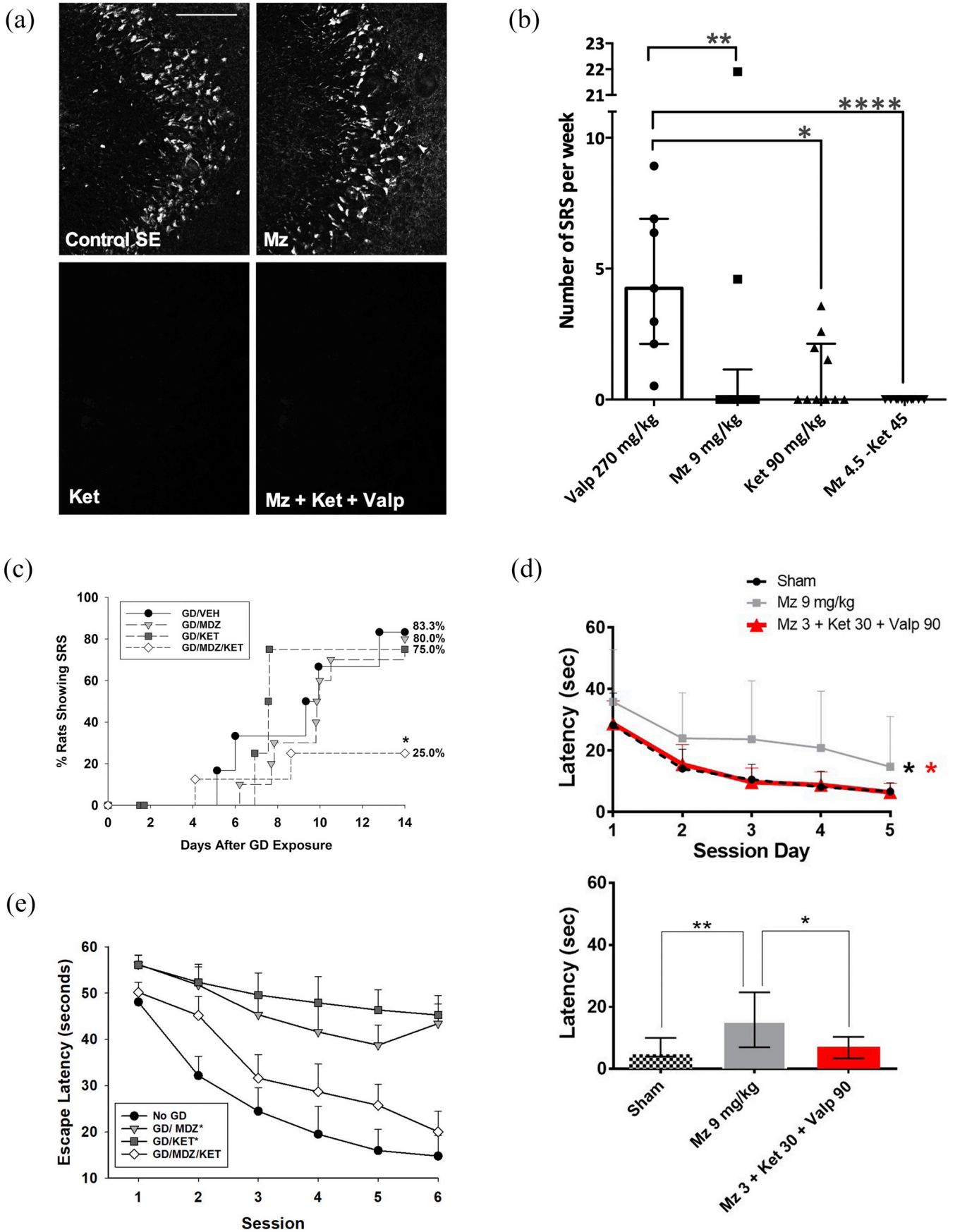
3.2.3.2. Spontaneous recurrent seizures. Fig. 3b shows that treatment with the midazolam + ketamine combination prevented epileptogenesis. No rat treated with that combination displayed any SRS (dual therapy: 0 SRS, n = 10, $p < 0.0001$ vs valproate controls). In the Tetz model, no untreated SE control survived long enough to be tested (6–8 weeks), so that we used as SE controls a group treated with valproate, which reduced mortality without changing the seizures in a measurable way. All rats which received 270 mg/kg of valproate, which increased long-term survival but did not alter the severity of SE, developed SRS (4.6 ± 1 SRS per week, n = 7). Some rats treated with double-dose ketamine developed SRS ($p < 0.05$ vs dual therapy) and so did some rats from the double-dose midazolam group, but differences with dual therapy were not significant. We do not know to what extent these results reflect a reduction in SE severity and duration versus a true antiepileptogenic action.

After soman-induced SE, 83.3% of untreated rats (VEH) that survived beyond 1 week (n = 5 of 6 survivors) developed SRS, versus 80% in the midazolam-treated group (n = 8 of 10 survivors) and 75% in the ketamine-treated group (n = 3 of 4 survivors). Treatment with the midazolam - ketamine combination (n = 2 of 8) had a 25% incidence of SRS (Fig. 3c). The percent of animals that developed SRS in the ketamine-midazolam group was significantly lower than the midazolam only group ($p < 0.05$).

3.2.3.3. Spatial memory deficit. In the Tetz model, SE-associated loss of spatial memory in the Morris Water maze (Morris, 1984) (Fig. 3d) was impaired in the valproate group (n = 7) compared to sham (no SE) controls (n = 8). Again, no untreated SE control survived long enough to be tested (6–8 weeks). In the acquisition test, the midazolam + ketamine group (n = 10) was undistinguishable from sham (no SE) controls and performed better than the valproate group (n = 7, $p < 0.0001$), the midazolam group (n = 10; $p < 0.05$) and the ketamine group (n = 10; $p < 0.01$). In the retention test, the valproate group was significantly different from the dual therapy and sham groups (which did not differ from each other) by Kruskal-Wallis.

In soman-induced SE (Fig. 3e), spatial memory deficits were evident in the groups treated with midazolam (GD/MDZ) or ketamine (KET/MDZ) monotherapy, while rats treated with a combination of midazolam and ketamine (GD/MDZ/KET) were not statistically different from untreated, no-SE controls (NO GD).

Altogether, these observations show that combining a GABAAR agonist and an NMDAR antagonist reduces not only the severity of SE but that of its long-term consequences as well.



(caption on next page)

Fig. 3. a) Untreated SE controls showed extensive neuronal injury in CA3, 48 h after SE. Animals treated with midazolam or valproate monotherapy showed no significant reduction of neuronal injury. Polytherapy with combinations of midazolam/ketamine or midazolam/ketamine/valproate at 40 min after seizure onset showed nearly complete protection from neuronal injury, but so did ketamine monotherapy. Results in CA1 were similar to CA3. In the dentate hilus, neuroprotection was less complete. Neuronal injury measured by unbiased stereology showed only an approximately 50% reduction in Fluoro-Jade-positive cells in all three ketamine-treated groups. *$p < 0.05$; **$p < 0.01$ by Kruskal-Wallis analysis followed by Dunn's test. b) Midazolam–ketamine dual therapy prevents epileptogenesis. Graph showing the number of SRS per week, ≥6 weeks after high-dose lithium-pilocarpine SE. The group treated with midazolam–ketamine dual therapy had no SRS. We used valproate monotherapy as our control group because of high mortality in the untreated control group. *$p < 0.05$, **$p < 0.01$ and ****$p < 0.0001$ versus valproate (Kruskal-Wallis followed by Dunn's test). From Niquet et al., 2016a, b. c) SRS after soman-induced SE. Treatment with the midazolam-ketamine combination reduced the incidence of SRS during the first 2 weeks after SE, compared to midazolam monotherapy ($p < 0.05$). d) Treatment of lithium-pilocarpine SE with the midazolam–ketamine combination reduces behavioral deficits. (Top) Performance in the Morris water maze shows the latency to reach the hidden platform (y-axis) on each testing day (x-axis). Acquisition is slower in the group treated with triple-dose midazolam monotherapy than in "no SE" controls. The group treated with midazolam + ketamine + valproate is indistinguishable from "no SE" controls. (Bottom) Latency to reach the hidden platform during the retention test also shows a significant reduction of latency in the combination therapy group compared to the triple-dose monotherapy group. **$p < 0.01$ versus valproate 270 mg/kg (by Kruskal-Wallis followed by Dunn's test). Data are presented as mean ± standard error of the mean (SEM). *$p < 0.05$ versus Mz4.5 + Ket45; **$p < 0.01$ versus Mz4.5 + Ket45 and ****$p < 0.0001$ versus Mz4.5 + Ket45 by two-way ANOVA. e) After sc Soman SE, spatial memory acquisition in the Morris Water Maze is slower in rats treated with midazolam (9 mg/kg) or ketamine (90 mg/kg) monotherapy than in controls which were not exposed to soman ("no GD") and had no seizures (*$p < 0.05$). In contrast, the combination of midazolam (3 mg/kg) and ketamine (30 mg/kg) was not significantly different from controls.

3.2.4. Drug selections based on the receptor trafficking hypothesis are more effective than guideline-recommended combinations of AEDs

3.2.4.1. Efficacy in stopping RSE. We wanted to examine whether a drug combination based on the receptor trafficking hypothesis, offered any advantage over drug combinations suggested by current evidence-based clinical guidelines, which recommend initial benzodiazepine monotherapy followed by an AED (e.g. fosphenytoin), then by another AED (e.g. valproate) or anesthesia (Glauser et al., 2016). We compared the effect of the midazolam 3 mg/kg-fosphenytoin 50 mg/kg-valproate 90 mg/kg combination (which follows AES guidelines) with the combination midazolam 3 mg/kg-ketamine 30 mg/kg-valproate 90 mg/kg, which targets seizure-induced changes in GABAA and glutamate receptors. Drugs were delivered simultaneously in both groups, 40 min after EEG seizure onset. The latter combination was far more effective in reducing the number of post-treatment seizures (Fig. 4A), the EEG power integral over the first hour post-treatment (Fig. 4B), the time needed for EEG amplitude to decline to twice the pre-seizure baseline (Fig. 4C) and the number of post-treatment spikes for 24 h (Fig. 4D).

3.2.4.2. Clinical relevance. The relevance of these results in rodents to the treatment of SE in humans is unknown. While receptor properties are similar in both species, and benzodiazepine pharmacoresistance has been observed clinically (Silbergleit et al., 2012; Treiman et al., 1998), circuitry and brain size are quite different in rats and humans, and the heterogeneity of clinical SE may limit the applicability of conclusions drawn from any animal model to clinical situations. However, the oncology experience has shown that drug dosage can be compared across species by using body surface area rather than body weight as a denominator (Pinkel, 1958; Guidance for Industry, 2005; Reagan-Shaw et al., 2008; Kaestner and Sewell, 2007). Comparing our 250–300 g rats to 70 kg humans by that method suggests that the doses of valproate, fosphenytoin and levetiracetam used in this study are not above the "human equivalent dose" (HED) derived from the clinical literature (Chen and Wasterlain, 2006; Gezalian et al., 2014; Glauser et al., 2016), while the midazolam dose used is slightly higher than the HED and the ketamine dose is three times the recommended HED (Chen and Wasterlain, 2006) but is lower than the dose used in patients with refractory SE who responded to ketamine (Gaspard et al., 2013). However, we have to remember that this method does not take into

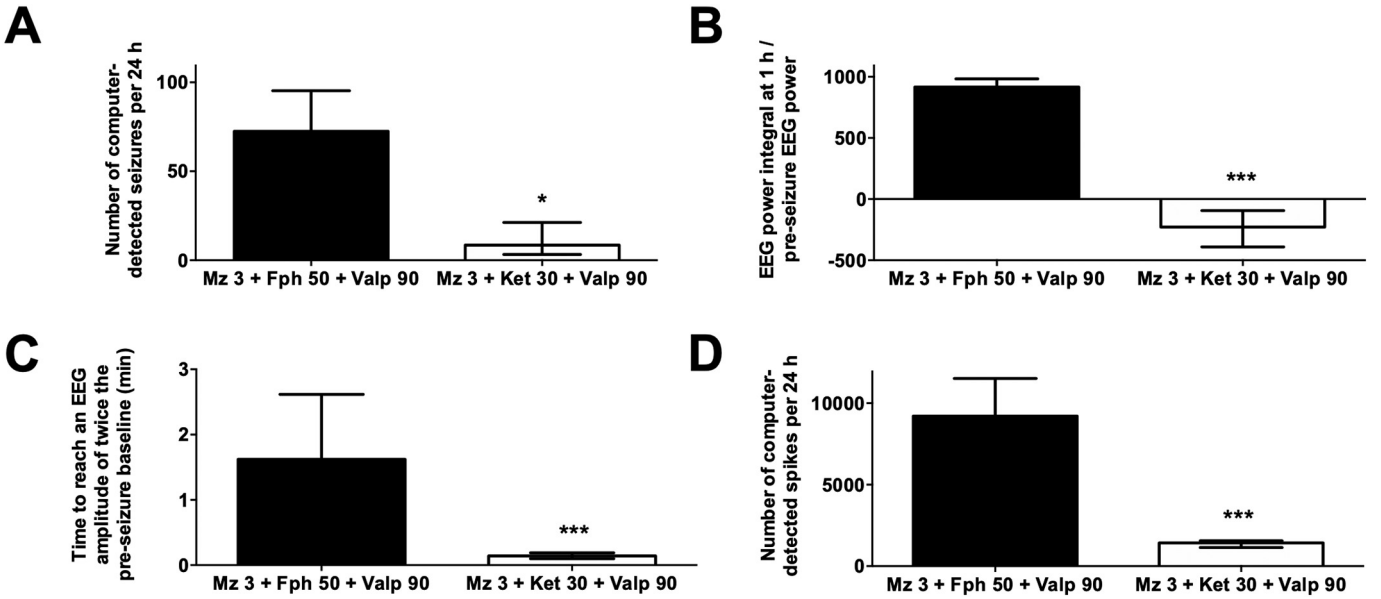


Fig. 4. The midazolam-ketamine-valproate combination, which targets seizure-induced changes in GABAA and glutamate receptors, is more effective that the midazolam-fosphenytoin- valproate combination, which follows American Epilepsy Society guidelines (Glauser et al., 2016). The graphs show the number of computer-detected seizures per 24 h (A), the ratio of EEG power integral over the first hour to initial EEG power at baseline (B), the time needed for EEG amplitude to decline from its very high value during SE to twice the pre-seizure baseline (C) and the number of computer-detected spikes per 24 h (D). The combination of 3 mg/kg midazolam, 30 mg/kg ketamine and 90 mg/kg valproate ($n = 10$) was more potent than the combination of 3 mg/kg midazolam, 50 mg/kg fosphenytoin and 90 mg/kg valproate. *$p < 0.05$, or***$p < 0.001$ by Mann-Whitney analysis. Fosphenytoin is expressed in Phenytoin Equivalents.

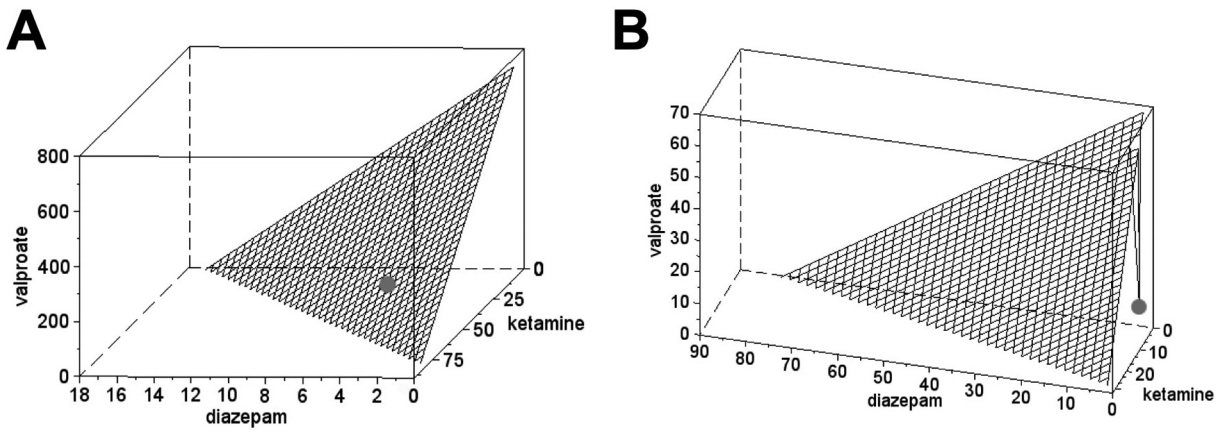


Fig. 5. Contribution of drug interactions to toxicity and efficacy. (A) Drug interactions do not contribute to the toxicity of the diazepam-ketamine-valproate combination. The measured toxicity of the drug combination (red dot) is exactly in the plane connecting individual drugs dosages producing a similar toxicity score (TD50's). Therefore, for the diazepam-ketamine-valproate combination, drug toxicity is neither synergistic nor antagonistic; it is simply additive. (B) Drug interactions contribute to the efficacy of the diazepam-ketamine-valproate combination. The efficacy of the three-drug combination in reducing the time needed for EEG power to fall to two times the pre-seizure baseline power, a good measure of seizure termination (red dot) is far below the plane connecting individual drug dosages producing a similar efficacy score (ED50's). Therefore, for the diazepam-ketamine-valproate combination, drug effects show strong positive cooperativity and are synergistic. This also means that the efficacy/toxicity ration of this combination is higher than the sum of its individual components, and its therapeutic index is improved by the use of this specific combination. From Niquet et al. (2017a, b). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

account differences in pharmacokinetics, and can provide a rough dose approximation at best.

3.2.5. Drug combinations based on the receptor trafficking hypothesis are synergistic and have a high efficacy/toxicity ratio

Isobolograms (Tallarida, 2000, 2006) suggest that the midazolam-ketamine-valproate combination potentiates the therapeutic response without potentiating drug toxicity (Fig. 5), so that the therapeutic index (Muller and Milton, 2012) is improved by switching from mono- to polytherapy. The key to therapeutic success is the ratio of efficacy to toxicity. If a combination of drugs potentiates both efficacy and toxicity, the latter will reduce the amount of drug tolerated, and nothing is gained. Fig. 5 shows that toxicity was simply additive between the three drugs of the combination, and that the therapeutic response was synergistic. As a result of that synergy, this three-drug combination delivered a greater therapeutic response than monotherapy for the same level of drug-induced toxicity. The mechanism of that synergy is unknown, since the drugs involved have no known direct interactions at the molecular level, and the effect is too rapid to be due to pharmacokinetic interactions. This synergism was powerful enough to overcome pharmacoresistance at the times tested in this study: the diazepam + ketamine + valproate combination stopped seizures at a time when pharmacoresistance to high doses of diazepam (20 mg/kg) was clearly present.

3.2.6. Timing of drug delivery is key

Standard practice in treating SE is sequential polytherapy, in which we wait for the first drug to fail before giving the second drug, and for the second drug to fail before giving the third drug (Glauser et al., 2016). This has the advantage of minimizing the number of drugs delivered to responders, but the disadvantage of delaying delivery of the second drug by at least 30 min., and delivery of the third drug by at least 1 h. The RAMPARTS study (Silbergleit et al., 2012) suggests that even small delays in treatment can result in differences in outcome. In order to mimic clinical situations where drugs are only injected after the previous treatment fails, we treated SE with the same drugs at the same dose in two groups of rats. In one group the three drugs were injected simultaneously. In the second group the second drug was injected 30 min after the first, and the third drug was delivered 30 min after the second drug (Fig. 6A). Simultaneous polytherapy was far more effective than sequential monotherapies in reducing the post-treatment EEG power integral during the first hour (Fig. 6B), or the first 6 h after treatment; in reducing the time needed for EEG amplitude to decline to twice the pre-seizure baseline (Fig. 6C) and in reducing the number of post-treatment seizures (not shown). By those measures, sequential monotherapies were not significantly different from high-dose benzodiazepine monotherapy, matching the clinical experience where the second and third drug used in sequential polytherapy have a low success rate (Treiman et al., 1998).

The far greater efficacy of simultaneous polytherapy over sequential monotherapies at the same dose is compatible with increasing pharmacoresistance, associated with seizure-induced increases in receptor trafficking, during the delay between sequential drug injections. The RAMPART trial (Silbergleit et al., 2012) showed that earlier treatment leads to better results, and that a brief gain of time (4 min.) can make a significant difference in outcome. Fig. 6 confirms that principle in the treatment of cholinergic RSE.

4. Conclusions

Traditional treatments of cholinergic seizures have been empirical, and fail to overcome pharmacoresistance when treatment is delayed. Our improving knowledge of the mechanisms of seizure-induced pharmacoresistance should prompt us to base treatment on pathophysiology. Cholinergic SE involves key changes in GABA and glutamate receptors, yet we continue to treat it with a single GABAergic drug, leaving changes in excitatory networks untreated. We also continue to delay administration of the second drug until the first one has failed, and administration of the third drug until the second one has failed, ignoring evidence that pharmacoresistance increases with time and with seizure burden. Replacing a benzodiazepine (diazepam) with another benzodiazepine with better pharmacokinetics (midazolam) is bound to fail because it does not address the main reason for benzodiazepine pharmacoresistance, which is the loss of synaptic GABAAR. Clinical trials comparing monotherapies are bound to show only minor differences between drugs, since each one treats only part of the problem, unless one practices “polytherapy” with a single drug which has multiple mechanisms of action (Krishnan et al., 2017).

We have tested the hypothesis that decreases in synaptic $GABA_AR$ and increases in synaptic glutamate receptors are key elements of the

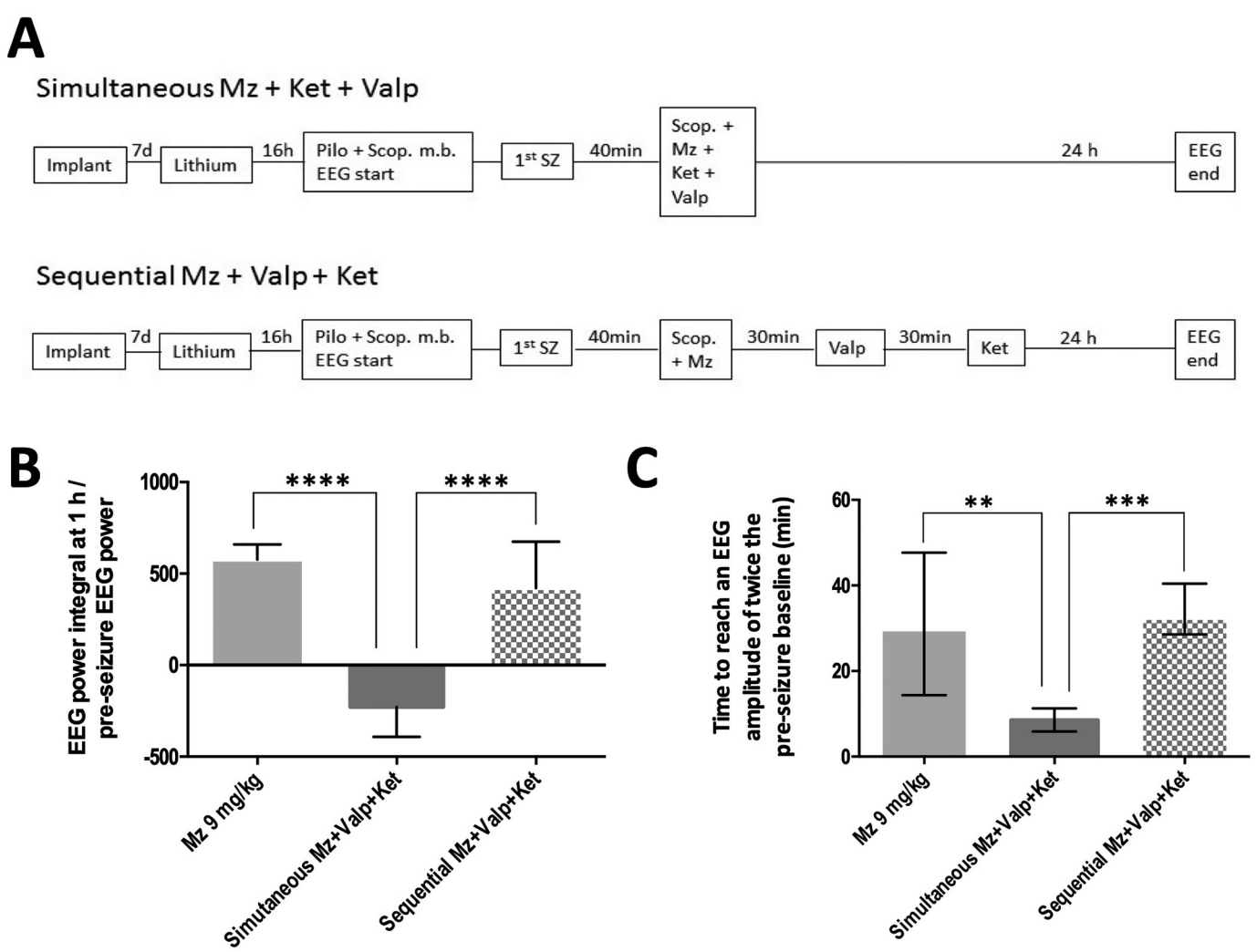


Fig. 6. Importance of the timing of drug delivery. A) Experimental flow: In the simultaneous group, the combination of midazolam 3 mg/kg, ketamine 30 mg/kg and valproate 90 mg/kg was administered simultaneously 40 min after SE onset. In the sequential group, the same drugs at the same dose were injected 30 min apart. B–C) The graphs show the ratio of EEG power integral to initial EEG power at baseline over the first hour (B), and the time needed for EEG amplitude to decline to twice the pre-seizure baseline without EEG bursts (C), indicating seizure termination. Simultaneous polytherapy (n = 10) was far more effective than sequential monotherapies (n = 8–9) or higher-dose midazolam (n = 10) in reducing EEG power, and in stopping SE. * $p < 0.05$, * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$ by ANOVA followed by Tukey's multiple comparison or by Kruskal-Wallis analysis followed by Dunn's test.

initiation and maintenance of cholinergic SE, and that both need to be treated to overcome seizure-induced pharmacoresistance when treatment is delayed. Our results support that hypothesis. In the treatment of severe benzodiazepine-refractory cholinergic SE, drug combinations which include a GABAAR agonist and a NMDAR antagonist are far more effective at stopping seizures than higher-dose monotherapy, or than other drug combinations, supporting a key role of GABAA and glutamate receptor trafficking in the pathophysiology of cholinergic SE. This is true in soman-induced SE as well as in a high-dose model of lithium-pilocarpine SE. These drug combinations not only terminate cholinergic RSE, but reduce or abolish its long-term consequences: neuronal injury, spatial memory deficits and epileptogenesis. Unlike benzodiazepines, they work when treatment is delayed by 40 min. Their seizure-stopping effects show positive cooperativity between drugs, so that their therapeutic index is improved by their synergistic interaction.

We compared this treatment to others inspired by standard clinical practice. Late treatment with the midazolam-ketamine-valproate combination was more potent than the midazolam-fosphenytoin-valproate combinations, showing that not all triple therapies show synergism, and suggesting that simultaneously targeting GABAAR and glutamate receptor changes is a valid therapeutic strategy. The simultaneous administration of the three drugs was far more efficient in stopping seizures than the standard practice of injecting the drugs sequentially, suggesting that timing of treatment is essential, and that an early polytherapy arm should be included in future clinical trials.

Declaration of Competing Interest

Jerome Niquet and Claude Wasterlain have a patent on polytherapy of cholinergic seizures (UC Case No. 2012-172-2). Other authors have no conflict of interest to disclose.

Acknowledgements

This work was supported in part by Merit Review Award# I01 BX000273-07 from the United States Department of Veterans Affairs (Biomedical Laboratory Research and Development Service), by NINDS (CounterACT grant UO1 NS074926) and by the Debbie and James Cho Foundation. We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

References

Bleck, T.P., 2006. Refractory status epilepticus. Curr. Opin. Crit. Care 11 (2), 117–120 Apr. (PMID: 15758590).

Chen, J.W., Wasterlain, C.G., 2006. Status epilepticus: pathophysiology and management in adults. Lancet Neurol. 5, 246–256.

Chou, T.C., 2006. Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies. Pharmacol. Rev. 58 (3), 621–681.

de Araujo Furtado, M., Zheng, A., Sedigh-Sarvestani, M., Lumley, L., Lichtenstein, S., Yourick, D., 2009 Oct 30. Analyzing large data sets acquired through telemetry from rats exposed to organophosphorous compounds: an EEG study. J. Neurosci. Methods 184 (1), 176–183. https://doi.org/10.1016/j.jneumeth.2009.07.020. (Epub 2009 Jul 24. PMID: 19632275).

de Araujo Furtado, M., et al., 2010. Spontaneous recurrent seizures after status epilepticus induced by soman in Sprague-Dawley rats. Epilepsia 51 (8), 1503–1510. https://doi.org/10.1111/j.1521167.2009.02478.x. Aug. (Epub 2010 Jan 7. PMID: 20067510).

DeLorenzo, R.J., et al., 1996. A prospective, population-based epidemiologic study of status epilepticus in Richmond, Virginia. Neurology 46, 1029–1035.

Dorandeu, F., et al., 2019. Protective effects of S+ ketamine and atropine against lethality and brain damage during soman-induced status epilepticus in guineapigs. Toxicology 234, 185–193.

Ferando, I., Mody, I., 2012. GABAA receptor modulation by neurosteroids in models of temporal lobe epilepsies. Epilepsia 53 (Suppl. 9), 89–101. https://doi.org/10.1111/epi.12038. Dec. (PMID: 23216582).

Fujikawa, D.G., 1996. The temporal evolution of neuronal damage from pilocarpine-induced status epilepticus. Brain Res. 725 (1), 11–22 Jun 24. (PMID: 882858e1).

Gaspard, N., et al., 2013. Intravenous ketamine for the treatment of refractory status epilepticus: a retrospective multicenter study. Epilepsia 54, 1498–1503.

Gezalian, M.M., et al., 2014. Management of Status Epilepticus. vol. 31. Federal Practitioner, pp. 24–29.

Glauser, T., et al., 2016. Evidence-based guideline: treatment of convulsive status epilepticus in children and adults: report of the guideline committee of the American Epilepsy Society. Epilepsy Curr. 16, 48–61.

Goodkin, H.P., et al., 2008. Subunit-specific trafficking of GABA(A) receptors during status epilepticus. J. Neurosci. 28, 2527–2538.

Guidance for Industry, 2005. In: FDA (Ed.), Estimating the Maximum Safe Starting Dose in Initial Clinical Trials for Therapeutics in Adult Healthy Volunteers.

Hjorth, B., 1991. Principles for transformation of scalp EEG from potential field into source distribution. J. Clin. Neurophysiol. 8, 391–396.

Holtkamp, M., et al., 2005. Predictors and prognosis of refractory status epilepticus treated in a neurological intensive care unit. J. Neurol. Neurosurg. Psychiatry 76, 534–539.

Joshi, S., Kapur, J., 2018. Mechanisms of status epilepticus: α-Amino-3-hydroxy-5- methyl-4-isoxazolepropionic acid receptor hypothesis. Epilepsia 59 (Suppl. 2), 78–81. https://doi.org/10.1111/epi.14482. Oct. Epub 2018 Aug 29. (PMID: 30159880).

Kaestner, S.A., Sewell, G.J., 2007. Chemotherapy dosing part I: scientific basis for current practice and use of body surface area. Clin. Oncol. (R. Coll. Radiol.) 19, 23–37.

Kapur, J., Lothman, E.W., 1990. NMDA receptor activation mediates the loss of GABAergic inhibition induced by recurrent seizures. Epilepsy Res. 5, 103–111.

Kapur, J., Macdonald, R.L., 1997. Rapid seizure-induced reduction of benzodiazepine and Zn2+ sensitivity of hippocampal dentate granule cell GABAA receptors. J. Neurosci. 17, 7532–7540.

Kapur, J., et al., 1989. Evidence that repetitive seizures in the hippocampus cause a lasting reduction of GABAergic inhibition. J. Neurophysiol. 61, 417–426.

Krishnan, J.K., et al., 2017. Brief isoflurane administration as a post-exposure treatment for organophosphate poisoning. Neurotoxicology 2017 (63), 84–89.

Lopez-Meraz, M.L., et al., 2010. Vulnerability of postnatal hippocampal neurons to seizures varies regionally with their maturational stage. Neurobiol. Dis. 37, 394–402.

Martin, B.S., Kapur, J., 2008. A combination of ketamine and diazepam synergistically controls refractory status epilepticus induced by cholinergic stimulation. Epilepsia 49, 248–255.

Mazarati, A.M., Wasterlain, C.G., 1999. *N*-methyl-D-asparate receptor antagonists abolish the maintenance phase of self-sustaining status epilepticus in rat. Neurosci. Lett. 265, 187–190.

Mazarati, A.M., et al., 1998a. Time-dependent decrease in the effectiveness of antiepileptic drugs during the course of self-sustaining status epilepticus. Brain Res. 814, 179–185.

Mazarati, A.M., et al., 1998b. Self-sustaining status epilepticus after brief electrical stimulation of the perforant path. Brain Res. 801, 251–253.

McDonough Jr., J.H., Shih, T.M., 1993. Pharmacological modulation of somaninduced seizures. Neurosci. Biobehav. Rev. 17, 203–215.

Mewasingh, L.D., et al., 2003. Oral ketamine in paediatric non-convulsive status epilepticus. Seizure 12 (7), 483–489 Oct. (PMID: 12967577).

Morris, R., 1984. Developments of a water-maze procedure for studying spatial learning in the rat. J. Neurosci. Methods 11 (1), 47–60.

Morrisett, R.A., et al., 1987. Effects of drugs on the initiation and maintenance of status epilepticus induced by administration of pilocarpine to lithium-pretreated rats. Exp. Neurol. 97, 193–200.

Muller, P.Y., Milton, M.N., 2012. The determination and interpretation of the therapeutic index in drug development. Nat. Rev. Drug Discov. 11 (10), 751–761.

Nakajima, T., et al., 1998. Epidemiological study of sarin poisoning in Matsumoto City, Japan. J. Epidemiol. 8, 33–41.

Nakamura, Y., et al., 2015. Regulation of GABAARs by phosphorylation. Adv. Pharmacol. 72, 97–146. https://doi.org/10.1016/bs.apha.2014.11.008.. Epub 2014 Dec 19. PMID: 25600368.

Naylor, D.E., Wasterlain, C.G., 2005a. GABA synapses and the rapid loss of inhibition to dentate gyrus granule cells after brief perforant-path stimulation. Epilepsia 46 (Suppl. 5), 142–147.

Naylor, D.E., Wasterlain, C.G., 2005b. Synaptic Non-NMDA Currents Increase in Dentate Gyrus Granule Cells During Status Epilepticus. vol. 2005 Society for Neuroscience Abstracts.

Naylor, D.E., et al., 2005. Trafficking of GABA(A) receptors, loss of inhibition, and a mechanism for pharmacoresistance in status epilepticus. J. Neurosci. 25, 7724–7733.

Naylor, D.E., et al., 2013. Rapid surface accumulation of NMDA receptors increases glutamatergic excitation during status epilepticus. Neurobiol. Dis. 54, 225–238.

Niquet, J., et al., 2016a. Midazolam-ketamine dual therapy stops cholinergic status epilepticus and reduces Morris water maze deficits. Epilepsia 57, 1406–1415.

Niquet, J., et al., 2016b. Benzodiazepine-refractory status epilepticus: pathophysiology and principles of treatment. Ann. N. Y. Acad. Sci. 1378, 166–173.

Niquet, J., et al., 2017a. Treatment of experimental status epilepticus with synergistic drug combinations. Epilepsia 58 (4), e49–e53. https://doi.org/10.1111/epi.13695. Apr. Epub 2017 Feb 22. PMID: 28225161.

Niquet, J., et al., 2017b. Simultaneous triple therapy for the treatment of status epilepticus. Neurobiol. Dis. 29 (104), 41–49 2017 Apr. (doi: 1016/j.nbd.2017.04.019. Epub 2017 Apr 29. PMID: 28461248).

Nissinen, J., et al., 2000. A new model of chronic temporal lobe epilepsy induced by electrical stimulation of the amygdala in rat. Epilepsy Res. 38 (2–3), 177–205 Feb. (PMID: 10642046).

Pinkel, D., 1958. The use of body surface area as a criterion of drug dosage in cancer chemotherapy. Cancer Res. 18, 853–856.

Rajasekaran, K., et al., 2012. Calcium-permeable AMPA receptors are expressed in a rodent model of status epilepticus. Ann. Neurol. 72 (1), 91–102.

Reagan-Shaw, S., et al., 2008. Dose translation from animal to human studies revisited. FASEB J. 22 (3), 659–661.

Rice, A.C., DeLorenzo, R.J., 1999. N-methyl-D-aspartate receptor activation regulates refractoriness of status epilepticus to diazepam. Neuroscience 93 (1), 117–123 (10430476).

Schultz, M.K., Wright, L.K., de Araujo Furtado, M., Stone, M.F., Moffett, M.C., Kelley, N.R., Bourne, A.R., Lumeh, W.Z., Schultz, C.R., Schwartz, J.E., Lumley, L.A., 2014 Jul-Aug. Caramiphen edisylate as adjunct to standard therapy attenuates soman-induced seizures and cognitive deficits in rats. Neurotoxicol. Teratol. 44, 89–104. https://doi.org/10.1016/j.ntt.2014.06.002. (Epub 2014 Jun 16. PMID: 24946037).

Sheth, R.D., Gidal, B.E., 1998. Refractory status epilepticus: response to ketamine. Neurology 51 (6), 1765–1766 Dec. (PMID: 9855547).

Shih, T., et al., 1999. Anticonvulsants for soman-induced seizure activity. J. Biomed. Sci. 6, 86–96.

Silbergleit, R., et al., 2012. NETT investigators. Intramuscular versus intravenous therapy for prehospital status epilepticus. N. Engl. J. Med. 366 (7), 591–600.

Suchomelova, L., et al., 2006. Treatment of experimental status epilepticus in immature rats: dissociation between anticonvulsant and antiepileptogenic effects. Pediatr. Res. 59, 237–243.

Tallarida, R.J., 2000. Drug Synergism and Dose-Effect Data Analysis. Chapman & Hall/CRC, BocaRaton, FL.

Tallarida, R.J., 2006. An overview of drug combination analysis with Isobolograms. J. Pharm. Exp. Therap. 319, 1–7.

Terunuma, M., et al., 2008. Deficits in phosphorylation of GABA(A) receptors by intimately associated protein kinase C activity underlie compromised synaptic inhibition during status epilepticus. J. Neurosci. 28 (2), 376–384. https://doi.org/10.1523/JNEUROSCI.4346-07.2008. Jan 9. (PMID: 18184780).

Tetz, L.M., et al., 2006. Development of a rat pilocarpine model of seizure/status epilepticus that mimics chemical warfare nerve agent exposure. Toxicol. Ind. Health 22, 255–266.

Treiman, D.M., et al., 1998. A comparison of four treatments for generalized convulsive status epilepticus. Veterans affairs status epilepticus cooperative study group [see comments]. N. Engl. J. Med. 339, 792–798.

Wasterlain, C.G., et al., 2000. Self-sustaining status epilepticus: a condition maintained by potentiation of glutamate receptors and by plastic changes in substance P and other peptides neuromodulators. Epilepsia 41 (S6), 134–143.

Zanos, P., et al., 2016. NMDAR inhibition-independent antidepressant actions of ketamine metabolites. Nature 533 (7604), 481–486. https://doi.org/10.1038/nature17998. May 26. Epub 2016 May 4. (PMID: 27144355).

Zanos, P., et al., 2018. Ketamine and ketamine metabolite pharmacology: insights into therapeutic mechanisms. Pharmacol. Rev. 70 (3), 621–660. https://doi.org/10.1124/pr.117.015198. Jul. Review. Erratum in: Pharmacol Rev. 2018 Oct;70(4):879. PMID: 29945898.